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Bacterial Scission of Ether Bonds

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INTRODUCTION

The ether linkage is the single most common and unifying structural feature which confers to both biological and xenobiotic compounds a high degree of resistance to biological mineralization. The ubiquitous distribution of the C—O—C molecular signature has considerable implications within the global biosphere. Monomers of lignin, the second most abundant organic polymer of natural origin, are bonded via intermonomer ether bridges, which contribute to the recalcitrance of this polymer. This intrinsic refractivity to biological destruction confines a significant portion of biospheric carbon in the form of lignin, which therefore constitutes a major component in the carbon cycle (131). Although we have benefited directly from the persistence of lignin within the environment, in that its long-term deposition ultimately leads to the formation of lignites and coals, the recalcitrance of some less innocuous C—O—C-containing compounds of industrial origin continues to be a source of appreciable concern.

Despite early recognition of the ether linkage as a major barrier to biodegradation (1), synthetic organic compounds eliciting a desired biological or chemical activity and possessing ether bridges still feature among pollutants released into the environment in vast quantities (73). Two distinct groups of xenobiotics which are regularly applied to soils and discharged into water courses and aquifers can be identified as the main offenders in this respect, namely, agrochemicals and detergents. With the continuing and ubiquitous discharge of ether compounds into aquatic and terrestrial environments, their persistence and subsequent toxicological effects are determined by the potential of the indigenous microflora to dissimilate them. In response to these omnipresent substances, a number of natural bacterial communities and isolates have evolved the capacity to degrade partially or wholly such xenobiotic compounds.

Microbial cleavage of the ether bond is a remarkable phenomenon, since the C—O bond energy is 360 kJ/mol (104) and necessarily demands an appreciable investment of energy to effect its fission in relation to the sometimes relatively small yield of assimilable carbon. A full analysis of this process of microbially mediated ether scission would involve (i) isolation of competent microorganisms; (ii) discovery of the precise mechanisms of ether cleavage by competent microorganisms and the purification and characterization of the constituent

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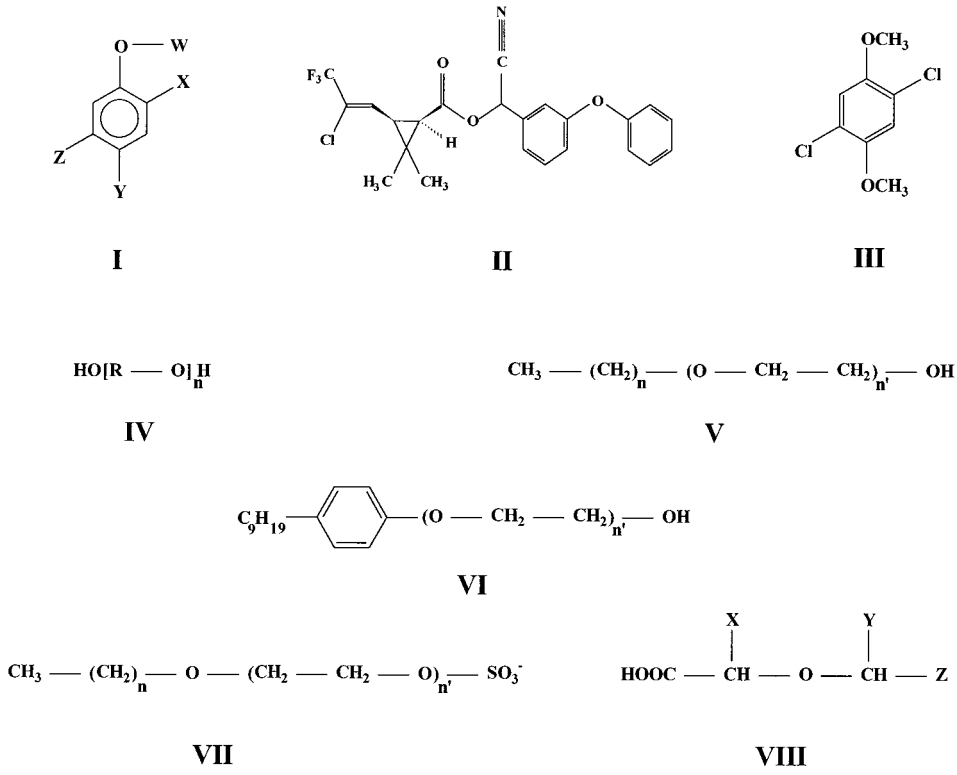

FIG. 1. I. Phenoxyalkanoic acid herbicides: (a) 2,4-dichlorophenoxyacetic acid (W = CH_2COOH, X = Cl, Y = Cl, Z = H), (b) 4-(2,4-dichlorophenoxy)butyric acid [W = $(CH_2)_3COOH$, X = Cl, Y = Cl, Z = H], (c) 2,4,5-trichlorophenoxyacetic acid (W = CH_2COOH, X = Cl, Y = Cl, Z = Cl), (d) 2-methyl-4-chlorophenoxyacetic acid (W = CH_2COOH, X = CH_3, Y = Cl, Z = H), (e) 2-methyl-4-chlorophenoxybutyric acid [W = $(CH_2)_3COOH$, X = CH_3, Y = Cl, Z = H], (f) dichloroprop [W = $CH(CH_3)COOH$, X = Cl, Y = Cl, Z = H], (g) mecoprop [W = $CH(CH_3)COOH$, X = CH_3, Y = Cl, Z = H], (h) fenoprop [W = $CH(CH_3)COOH$, X = Cl, Y = Cl, Z = Cl]. II. Cyhalothrin. III. Chloroneb. IV. Polyethers: (a) PEGs (R = CH_2CH_2), (b) PPGs [R = $CH(CH_3)CH_2$], (c) PTMGs [R = $(CH_2)_4$]. V. Alcohol ethoxylate surfactants (n = 5 to 25; n' = 5 to 25). VI. Nonylphenol ethoxylate surfactants (n' = 5 to 25). VII. Alkylethoxy sulfate surfactants (n = 5 to 25, n' = 5 to 25). VIII. Organic builder compounds: (a) oxydiacetate (X = H, Y = H, Z = COOH), (b) CMOS (X = H, Y = CH_2COOH, Z = COOH), (c) lactylsuccinate (X = CH_3, Y = CH_2COOH, Z = COOH), (d) ethylene glycol diacetate (X = H, Y = H, Z = CH_2OCH_2COOH).

ether-cleaving enzyme(s); (iii) isolation and sequence analysis of genes encoding the biodegradative enzymes; (iv) identification of substrate structural features which dictate the capacity of microorganisms to cleave the C—O—C bond, thereby permitting the design of compounds which exhibit a desired biological and chemical activity and yet are more accessible to biodegradation; and (v) engineering and selection of new strains of microorganisms tailored to cleave ether linkages more effectively.

The dual purpose of this article is first to review the range of environmental ether-containing pollutants and related compounds and their susceptibility to bacterial biodegradation and second to detail the known biochemical mechanisms of ether scission reactions present in bacteria which act on these substances.

In the past, enzymes involved in the bacterial scission of ether bonds have been referred to as etherase enzymes (see, e.g., reference 125). By analogy with other trivial names for enzymes (phosphatase, glycosidase, etc.), this name implies catalysis of a hydrolytic scission of the substrate. However, scrutiny of the available evidence suggests not only that bacterial scission of ether bonds is catalyzed by a heterogeneous group of enzymes or enzyme systems exhibiting a variety of mechanisms but also that enzymic hydrolytic ether cleavage is a very minor contributor, if indeed it truly occurs at all. Thus, although "etherase" is a convenient expression, in this review we have avoided its use.

In terms of the capacity to attack and mineralize ether-containing xenobiotics, microbial communities appear far superior to axenic cultures (33, 64, 91, 106). While mixed-culture biocatalysts justly deserve mention, their detailed examination here is not germane to the emphasis of this review.

PROBLEM POLLUTANTS AND ETHER SCISSION

Agrochemicals

A perfunctory examination of *The Agrochemicals Handbook* (60) shows the large number of agrochemicals which possess an ether linkage. Apart from the active ingredient, additional ether-containing constituents such as detergents (see below) may be included in these formulations (51). Herbicides, insecticides, fungicides, and rodenticides represent a major source of pollutants, whose persistence in the environment may cause a deleterious perturbation in the delicate balance of the ecosystems to which they are applied. Invariably, they exert an undesirable toxic or harmful effect upon other organisms in addition to the intended target. Few agrochemicals are completely refractive to biodegradation, yet certain compounds, particularly the ether-containing members, are more slowly degraded and therefore persist in the environment for longer periods. A protracted residency is more often a consequence of environmental factors uncongenial to bacterial growth and metabolism in tandem with the physicochemical conditions dictating the rate of biodegradation, rather than to a poor intrinsic

capacity of the indigenous microflora to perform the process (2, 102).

The phenoxyalkanoic acid group of herbicides (Fig. 1, structure I) are a set of auxin analog growth regulators which are particularly effective against broad-leaved weeds. Although long employed as organic herbicides, these compounds are only slowly biodegraded and tend to evoke severe toxicological problems (42). Not surprisingly, microbial degradation of the phenoxyalkanoic acids has been the focus of considerable attention: at least 16 species of bacteria, including *Corynebacterium*, *Nocardia*, *Achromobacter*, *Arthrobacter*, and *Flavobacterium* species, have been identified as proficient biocatalysts mediating the degradation of phenoxyacetic acids (44). *Arthrobacter* (66) and *Pseudomonas* (24) species which are particularly adept at degrading 2,4-dichlorophenoxyacetic acid (Fig. 1, structure Ia) have been isolated. The destruction of herbicidal activity was accomplished in both cases by cleavage of the ether linkage between C-2 of the alkanoic acid and C-1 of the aromatic portion of the molecule (24, 67, 117). By employing phenoxy-[^{18}O]acetic acid, Helling et al. (43) illustrated that whole cells or cell extracts of an *Arthrobacter* species cleaved the ether linkage joining the ether oxygen to the alkanoic side chain. MacRae et al. (68) described a cognate cleavage of 4-(2,4-dichlorophenoxy)butyric acid (Fig. 1, structure Ib) with a pure *Flavobacterium* strain in which 2,4-dichlorophenol and butyric acid were the products. From the perspective of bioremediation, these biocatalysts are of limited use on their own, since this intramolecular scission generates substituted phenols, which are themselves intrinsically toxic substances.

The compound 2,4,5-trichlorophenoxyacetic acid (Fig. 1, structure Ic) is particularly effective against woody plants, and its half-life in soil suspensions (14 days) exceeds that of 2,4-dichlorophenoxyacetic acid (4 days). Kilbane et al. (61) reported that this compound was degraded by a strain of *Pseudomonas cepacia* which cleaved the ether bond and also generated chlorinated phenol as an end product. Two herbicides structurally related to 2,4,5-trichlorophenoxyacetic acid are 2-methyl-4-chlorophenoxyacetic acid and 2-methyl-4-chlorophenoxybutyric acid (Fig. 1, structures Id and e), which are used extensively as herbicides, are considerably less toxic than 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid, but their residual activity remains in the environment for approximately 4 months and 6 weeks, respectively (60). Biodegradation and growth on 2-methyl-4-chlorophenoxyacetic acid as a carbon source have also been described for a number of organisms including a *Pseudomonas* species (31). This organism also carries out initial dissimilation via an ether cleavage reaction (29). Acquisition by weeds of tolerance to 2,4-dichlorophenoxyacetic acid and the structurally similar 2-methyl-4-chlorophenoxyacetic acid has encouraged the use of 2-phenoxypropanoic acid herbicides such as dichloroprop (Fig. 1, structure If), mecoprop (structure Ig), and fenoprop (structure Ih). The herbicidal activity of mecoprop persists in the environment for approximately 2 months after application and is eliminated by microbial cleavage of the ether linkage.

Diphenyl ether herbicides and insecticides are used extensively as pest control agents and, like the pesticides discussed above, represent a serious problem of environmental concern. The recalcitrance of chlorinated diphenyl ethers has led to their accumulation within food chains (76, 81), which, in the light of the discovery that certain diphenyl ethers such as lactofen (a halogenated nitrodiphenyl ether herbicide) can induce tumours in mice (9), is a source of some anxiety. Cyhalothrin (Fig. 1, structure II), a diphenyl ether-based insecticide, is biodegraded within 4 to 12 weeks of application, but the process reputedly involves the hydrolysis of the ester bond and thus generates a product with the ether linkage intact. The ability to cleave the ether linkage of a diverse range of diphenyl ethers has been demonstrated for several bacteria, including *Pseudomonas* (88, 127), *Erwinia* (105), and *Sphingomonas* (97) species. These biodegrading organisms must have evolved mechanisms of ether scission which defy the steric hindrance of the diphenyl ether structure.

Chloroneb (Fig. 1, structure III), a chlorinated aryloxy compound, has been used as a fungicide to control *Pythium* and *Rhizoctonia* infection; although it is recognized as a negligible hazard according to the Environmental Protection Agency and the World Health Organization, it has a half-life in soil of 3 to 6 months. Microbial attack on, and inactivation of, this compound also occur at the site of the ether substituent by means of an O-demethylation reaction (45).

Polyethers

Polyethylene glycols (PEGs), polypropylene glycols (PPGs), and polytetramethylene glycols (PTMG) constitute a group of closely related and widely used polyether compounds (Fig. 1, structure IV). Chemically unsubstituted PEGs are used in a wide range of applications including antifreeze formulations, water-soluble lubricants, wetting agents, pharmaceutical preparations, and cosmetics. In addition, covalent attachment of PEGs to hydrophobic groups such as long-chain alkyl groups, alkylphenol groups, or acyl chains, either directly or via other moieties such as sorbitol, generates nonionic surfactants, a very important group of industrial products with applications from domestic detergents to agrochemicals and food emulsifiers. While free and substituted PEGs undergo biodegradation through cleavage of ether bonds, the presence of the hydrophobic substituents in the surfactants adds further complexity to the biodegradative pathways, thereby justifying separate consideration in the subsequent section.

Polyethylene glycols. Since the initial isolation of a bacterium capable of growth at the expense of ether glycols (25), a host of aerobic and anaerobic bacteria with the capacity to grow on low-molecular-weight ethylene glycols (molecular weight, 62 to 1,500) and exhibiting mechanisms of ether cleavage have been documented (12, 36, 48, 79, 120, 121). In addition, there are several reports of analogous dissimilation of higher-molecular-weight PEGs (molecular weight, up to 20,000) by bacteria (39, 77, 95). A strain of *Pseudomonas aeruginosa* isolated by Haines and Alexander (39) degraded high-molecular-weight PEG, reputedly by means of a depolymerizing, hydrolytic, extracellular enzyme. Whole cells converted PEGs to a mixture of mono-, di-, and oligomers of ethylene glycol, which were subsequently used as substrates for growth. A similar extracellular location was proposed for the ether scission of PEGs by a *Bacteroides* sp., in which initial cleavage of the interior bonds also resulted in the generation of oligomeric intermediates (21). However, these results have never been confirmed, and the evidence for an extracellular location for the depolymerizing enzyme(s) and for a hydrolytic mechanism remains circumstantial and unsubstantiated. In contrast to these two organisms, a strain of *Pseudomonas stutzeri* examined by Obradors and Aguilar (77) exhibited considerably higher rates of biodegradation of PEGs (molecular weight, up to 14,000) reportedly accomplished via the activity of a periplasmic dehydrogenase. On the basis of this cellular localization and the upper limit on the size of PEGs capable of supporting growth, these authors suggested that porins might facilitate the transport of the PEG across the outer membrane

into the periplasmic space. In contrast, ether cleavage of high-molecular-weight PEG is confined to the cytoplasm in *Pelobacter venetianus* (28), but an explanation to account for the transit of PEG across the inner membrane is lacking and there is some doubt concerning the effectiveness of porins, isolated from the outer membrane of this organism and examined in reconstituted lipid bilayers, to transport PEG (96). A point raised by Dwyer and Tiedje (21) concerns the structural conformation of PEG molecules; although the lower-molecular-weight PEGs display a zig-zag shape, the larger polymers display a helical conformation which could directly influence the uptake and depolymerization processes.

Polypropylene glycols and polytetramethylene glycols. Kawai et al. (55) isolated from soil and activated sludge 53 strains of bacteria each endowed with the capacity to utilize PPG 2000 or PPG 4000 as carbon and energy sources. Strain no. 7 (a *Corynebacterium* strain) displayed the greatest growth yield on PPG (PPG 670 to PPG 4000) but could not assimilate a similar size range of PEG (PEG 400 to PEG 6000). Depolymerization of PPG by this organism was attributed to intracellular enzyme(s) effecting cleavage of ether bonds. In contrast to the microbial biodegradation and assimilation of PEG and PPG, few bacteria with the ability to degrade and grow on PTMG have been isolated. Kawai and Yamanaka (59), however, reported the enrichment of two bacteria, *Alcaligenes denitrificans* subsp. *denitrificans* and *Pseudomonas maltophilia*, each of which was proficient at degrading and growing on PTMG 265 and PTMG 200.

Detergents

Modern commercial and household detergents are cocktails of organic and inorganic compounds with disparate structures, functions, and resistances to biodegradation. Most detergent formulations contain 10 to 20% surface-active agent (surfactant), with the bulk of the remainder consisting of a "builder" component. The environment receives continuous input of copious amounts of domestic and industrial detergents, a high proportion of which consists of ether-containing nonionic (Fig. 1, structures V and VI) and anionic (structure VII) surfactants. The current sales value of nonionic surfactants constitutes almost half that for all surface-active agents, with a projected annual world market increase of 5.4%, reaching 1.4 billion dollars in 1997 (101). These figures indicate the continuing commercial exploitation of nonionic surfactants and a potentially increasing burden upon the environment. Apart from their obvious employment as cleansing agents in detergent formulations, alcohol ethoxylates (Fig. 1, structure V) and alkylphenol ethoxylates (structure VI) are also used in a remarkable diversity of applications including incorporation into agrochemical formulations as dispersants (51). Many biodegradation studies have focused on the use of mixed populations of sewage organisms and naturally occurring assemblages of river water bacteria to facilitate the primary biodegradation of alcohol ethoxylates and alkylphenol ethoxylates (10, 11). This has allowed a prognosis of the fate of these substances in the environment yet lends little to the identification and understanding of organism-specific steps and the mechanism of the biodegradation process.

Most of the problems associated with the release of detergent formulations into the environment have arisen as a consequence of the surfactant and to a lesser extent the builder component. Although surfactants in general have previously been appraised as being "apparently" nontoxic to humans when present at concentrations of 1 ppm (112), more recent evidence strongly indicates the contrary for particular surfactants (126). The commonly recognized problems associated with release of surfactants into the environment via water courses and aquifers are manifold: aside from the esthetically undesirable problem of foaming in rivers and drinking water (at levels below 1 ppm), retention of surface-active properties can cause difficulties in the wastewater treatment process (113). Moreover, the biological mineralization of surfactants can severely increase the biological oxygen demand in water courses, with pernicious consequences (73). Herein lies a dilemma; on the one hand, high levels of recalcitrance result in specific problems arising from retention of surface activity, while on the other hand, a rapid biodegradation of surfactants may seriously diminish oxygen availability to aquatic species. Resolution of this problem might be satisfied by the utilization of surfactants which readily undergo primary degradation and thereby lose their surface-active properties but are further biodegraded at a lower rate so as not to increase radically the biological oxygen demand. This will of course generate intermediates of surfactant primary biodegradation which may themselves have adverse effects upon ecosystems.

Builder compounds. In contrast to surfactants, the release of builder compounds into the environment provokes fewer and less dramatic problems. Recognition in the 1970s that both nitrogen- and phosphorus-containing builder compounds were encouraging the eutrophication of aquatic environments (65) initiated the exploration for organic alternatives (8). Although most potential organic builders, many of which contained ether linkages, were precluded from commercial application for a number of reasons (86), a detailed examination of their recalcitrance provided further insight into the mechanisms of microbially mediated ether cleavage. A study concentrating on 18 structurally similar potential organic builder compounds indicated that only oxydiacetate (Fig. 1, structure VIIIa), carboxymethyloxysuccinate (CMOS; structure VIIIb), lactylsuccinate (Me-CMOS; structure VIIIc), and ethylene glycol diacetate (structure VIIId) were readily biodegradable (35). The mechanism of degradation of one of these sequestrants, CMOS, was first elucidated by Peterson and Llaneza (87), who showed that an inducible lyase present in a *Zoogloea* sp. cleaved the ether bond to form glycolate and fumarate as the immediate products. Cain (11) surmised that the seemingly universal capacity of microorganisms to degrade CMOS is explained by the combined activities of an active uptake system and an endogenous lyase, which are coinduced by CMOS and fortuitously accept it as a substrate.

Alcohol ethoxylates. The alcohol ethoxylate surfactants consist of long alkyl chains, usually containing 5 to 25 carbon atoms, which may be alkyl branched, covalently linked to a hydrophilic ethoxylate chain, generally composed of 5 to 25 ethylene oxide units. Thus, by analogy with the biodegradation of unsubstituted PEGs and irrespective of any structural diversity in the hydrophobic components, these surfactants may undergo biodegradation by scission of ether bonds in the PEG moiety. There is abundant evidence (124) for the occurrence in mixed cultures of exo-cleavage of terminal C_2 units and endo-cleavage to yield oligomeric glycols. Exo-cleavage of the ether linkage and sequential removal of ethoxy units from linear alcohol ethoxylates has also been observed in pure culture and is a feature common to several anaerobic microorganisms such as *Pelobacter propionicus*, *Acetobacterium* sp. (120), and *Pelobacter venetianus* (95). In contrast to aerobic organisms, these cultures metabolize the hydrophilic cleavage product whereas the hydrophobic alkyl residue is left to accumulate in the form of the equivalent fatty acid.

From a bacterial nutritional perspective, nonionic surfactants provide carbon for energy and growth, not only in the

PEG but also in the hydrophobic alkyl moiety. Direct access to the alkyl chains can be achieved by separation of hydrophobic groups from PEG by cleavage at the alkyl-ether bond, often referred to as central ether cleavage (124, 125). The alkyl chain, whether liberated as alcohol, aldehyde, or carboxylic acid, is readily assimilated by central metabolic pathways. Watson and Jones (122) demonstrated that in *Pseudomonas* sp. strain SC25A, central cleavage of dodecyl decaethoxylate was the preferred form of attack. The process was rapid, liberated CO_2 via β-oxidation of the alkyl chain, and generated decaethoxylate, which accumulated. In addition, Ichikawa et al. (47) reported the liberation and accumulation of PEG during growth of an axenic culture of a (putative) *Pseudomonas* sp. on alcohol ethoxylates. Central ether cleavage was also reported as the predominant mechanism of primary biodegradation of alcohol ethoxylates by a strain of *Acinetobacter lwoffii* (98). In some cases, e.g., the initial degradation of triethyleneglycol dodecylether by *Pseudomonas* strain DES1, the ether cleavage system shows poor regioselectivity with regard to the exact site of ether scission (37, 38), so that central ether cleavage and PEG endo-cleavage occur simultaneously.

For completeness, it is worth noting that a third route contributing to the biodegradation of linear alkyl ethoxylates involves ω/β-oxidation of the alkyl chain in the intact surfactant (reference 124 and references therein) to yield short-chain carboxylic acids ω-attached to PEGs. Extensive branching in the alkyl chain is strongly inhibitory to β-oxidation and deflects bacterial attack to central (alkyl-)ether cleavage and exo-/endo-degradation of the PEG moiety (124). Moreover, branching close to the central ether hinders scission at this site (122) and results predominantly in PEG biodegradation.

From the foregoing comments, it is clear that both the hydrophobic and hydrophilic moieties of linear alcohol ethoxylates can influence biodegradability and toxicity (83). In short, biological decomposition of these nonionic surfactants is markedly accelerated by the linearity and a reduced size of the alkyl chain and by a lower degree of ethoxylation (113). For PEG dodecyl ether, the rates of primary biodegradation and mineralization by three strains of bacteria have been shown to decrease substantially when three or more ethylene oxide units are present in the molecule (116). Thus, for linear alcohol ethoxylates of short PEG content, biodegradation focuses on access to and metabolism of the alkyl chain. Conversely, when the alkyl chain is highly branched, its metabolism is hindered and biodegradation of the surfactant is effected by ether-cleaving reactions in the PEG.

Alkylphenol ethoxylates. Although the linear alcohol ethoxylates and their degradation products are apparently harmless in low concentrations (119), recent data suggest that their alkylphenol equivalents may pose a significant human threat (126). Whereas alcohol ethoxylates are biodegraded in the environment within 1 or 2 weeks, the alkylphenol ethoxylates persist in the environment for considerably longer (113, 124). Inclusion of an aryl substituent in the ethoxylate structure sterically hinders central ether scission and retards the rate of primary biodegradation but does not appear to protect other ether linkages from attack. Over a period of 7 days, a *Nocardia* sp. generated all the lower homologous ethoxylates from phenol hexaethoxylate and 4-*n*-butylphenol hexaethoxylate (4). Clearly, primary biodegradation of this molecule was achieved by sequential shortening of the glycol chain. To corroborate these data, the primary mechanisms of nonylphenol ethoxylate degradation have been elucidated for *Pseudomonas* species: a progressive shortening of the hydrophilic chain by single ethoxy units was observed, leaving nonylphenol diethoxylate and nonylphenol ethoxy acetic acid as nondegradable products

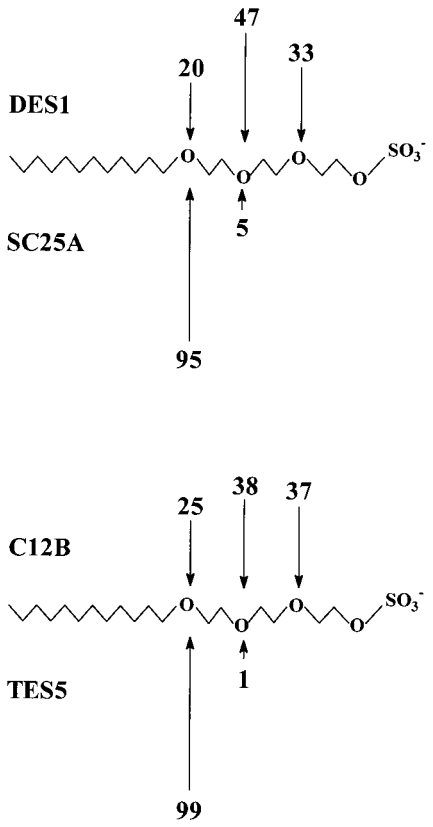

FIG. 2. Regioselectivity of the ether-cleaving activities in four bacteria which biodegrade alkyl ethoxylate and alkylethoxy sulfate surfactants. Arrows indicate the point of cleavage, and their lengths indicate the relative percentages of the substrate ether bond cleaved at that position (values quoted at base of arrow).

(32, 69). The significance of these findings is manifest by the accumulating evidence which substantiates the association of serious toxicological problems with nonylphenol ethoxylate biodegradation products (34, 49, 74, 103). These facts, in tandem with findings suggesting that surfactant derivatives are bioaccumulable within elements of food chains (22, 72), form a cogent argument against the commercial employment and release of alkylphenol ethoxylates into the environment.

Alkylethoxy sulfate surfactants. Primary biodegradation of alkylethoxy sulfate (Fig. 1, structure VII) members of the anionic group of surfactants involves mainly ether cleavage (41, 125). *Pseudomonas* SC25A and strain TES5 showed very strong preference for ether scission of sodium dodecyl triethoxysulfate compared with the alternatives of sulfate removal from the hydrophilic end of the molecule or ω/β-oxidation of the hydrophobic dodecyl chain (41). Moreover, of the three ether bonds in the molecule, these strains attacked the central ether linkage with strong regioselectivity (Fig. 2). In contrast, *Pseudomonas* sp. strains C12B and DES1 showed appreciable sulfatase activity as well as ether scission. These strains were also much less specific for cleavage of the alkyl-ether bond and showed higher activity toward the PEG-ether linkages than toward the central ether bond (Fig. 2). The invariability of this pattern of ether bond specificity with growth conditions for strain DES1 was interpreted (40) as evidence for a single ether-cleaving enzyme; however, so far, definitive proof for a single enzyme has not been obtained.

BIOCHEMICAL MECHANISMS OF ETHER CLEAVAGE

Cain (11) listed four principal mechanisms as the main biochemical strategies that might be adopted by microorganisms to attack ether linkages: (i) oxygenative cleavage via monooxygenases; (ii) oxidation of the carbon atom α-linked to the ether bond, followed by hydrolysis of the resultant ester; (iii) direct hydrolysis of the C—O bond; and (iv) carbon-oxygen lyase-mediated cleavage. Since that summary by Cain and a subsequent discussion of the subject by Kawai (54), several additional mechanisms of microbial ether cleavage have been proposed, necessitating a reappraisal of all possible modi operandi of ether scission. A contemporary catalog of the means of microbial ether attack includes (i) oxygenases; (ii) cytochromes P-450; (iii) hydroxyl shift; (iv) hydrolysis; (v) anaerobic cleavage of methyl-aryl ethers; (vi) oxidative mechanisms; (vii) reductive mechanisms; and (viii) carbon-oxygen lyases. Current knowledge of these is now described.

Oxygenation

The simplest ether, dimethyl ether, has been shown to be degraded by resting cells, cell extracts, and the purified methane monooxygenase of *Methylococcus capsulatus* (Bath) (107). The proposed mechanism for ether scission, based on the detection of formaldehyde and methanol as reaction products, is that the monooxygenase hydroxylates one of the methyl groups by insertion of an oxygen atom, to yield the hemiacetal methoxy methanol. Hemiacetals are unstable in aqueous solution and spontaneously dismutate into alcohol and aldehyde. Thus, the actual cleavage step itself is not enzyme catalyzed; the role of enzyme(s) in this and many other cases, as we shall see, is to labilize the ether by converting it to a structure which is predisposed to spontaneous scission.

When the symmetry in a dialkyl ether is replaced by the asymmetry of aryl-alkyl ethers, the prospects exist, at least in principle, for either aryl-O or alkyl-O cleavage. The aryl-O bond is generally stronger than the alkyl-O bond because of conjugation of oxygen *p*-orbitals with the aromatic π-electron system. Thus, from a chemical standpoint, alkyl-O cleavage might be expected to dominate, and this is indeed the case. Early results (14) showing the ability of *Pseudomonas fluorescens* to convert vanillate (3-methoxy-4-hydroxybenzoate) to formaldehyde and the corresponding demethylated phenol, protocatechuate, were later confirmed for *P. aeruginosa* (89). Similarly, acetaldehyde was the product of O deethylation of 4-ethoxybenzoate in *Nocardia* species (14). These product identities and the utilization of NADH and O_2 in stoichiometric amounts (89) supported the interpretation that the reaction was a monooxygenation. Although not explicitly proposed by Ribbons (89), the mechanism presumably is the same as that occurring in the demethylation of dimethyl ether, namely, hydroxylation to the unstable hemiacetal. Studies on a parallel system in *P. testosteroni* (90) indicated a similar route but with a broader specificity of demethylase activity, which demethylated not only vanillate but also both methyl groups in veratrate (3,4-dimethoxybenzoate). Moreover, methyl substituents attached directly to the aryl ring were also oxidized (but not necessarily cleaved) in *Pseudomonas* spp. (90), thus supporting the methyl hydroxylation mechanism.

The enzymes involved in the methyl hydroxylation appear to be two-component systems (7, 90). Bernhardt et al. (7) described a novel monooxygenase in *Pseudomonas putida* which oxidatively demethylated 4-methoxybenzoate to 4-hydroxybenzoate and formaldehyde. The responsible enzyme, 4-methoxybenzoate monooxygenase (EC 1.14.99.15) from *P. putida* DSM 1868, was identified as consisting of two components, a 42-kDa NADH-dependent reductase and a 120-kDa monooxygenase. This enzyme demethylated 4-methoxybenzoate with stoichiometric amounts of NADH and O_2 by inserting one atom from molecular oxygen into the C—H bond of the methoxy group and reducing the second atom of oxygen to water. The immediate hemiacetal product decayed spontaneously to 4-hydroxybenzoate and formaldehyde (6). Thus, broad consensus has emerged that oxygenase enzymes initiate the scission of dimethyl ethers and methyl-aryl ethers by hydroxylation through oxygen insertion at a methyl group to generate the unstable R-O-CH_2-OH hemiacetal structure.

In contrast to dialkyl and alkyl-aryl ethers, the ether cleavage of diaryl ethers offers no choice but to break an aryl-O bond. Nevertheless, in the bacterial degradation of some diaryl ethers, the hemiacetal structure, formed through the agency of oxygenase activities, again features as the key precursor to ether cleavage. However in these cases, dioxygenation rather than monooxygenation is the rule. Thus, on the basis of identification of metabolites, measured enzyme activities, and ^{18}O-labeling studies (97), the degradation of 3-methyldiphenyl ether in *Sphingomonas* species is considered to proceed via dioxygenation at any one of the three different aryl C—C bonds adjacent to the ether linkage (Fig. 3). In each route, an ether-linked carbon is converted to a hemiacetal structure, which breaks down spontaneously to the observed mixture of phenolic and catecholic products. A comparable mechanism based on similar evidence was proposed for degradation of 3- and 4-carboxybiphenyl ethers in *Pseudomonas* sp. strain NSS2 (127).

The mechanism of diphenyl ether cleavage in *P. cepacia* appears to be more complex, because an early intermediate in the pathway was 2,3-dihydroxydiphenyl ether, formed by dioxygenation (or possibly sequential monooxygenation) but not adjacent to the ether linkage (88). Thus, no hemiacetal was formed. It was proposed that ether scission most probably occurred following a further oxygenation, this time with simultaneous cleavage of the ether. However, the details of this mechanism and the evidence to support it have yet to be found.

From the foregoing discussion, it emerges that bacterial biodegradations of dialkyl, alkyl-aryl, and diaryl ethers may all be initiated through the action of oxygenase enzymes. Without exception, the available evidence shows that the role of these enzymes in ether degradation is not the scission of the C—O—C linkage per se but, rather, the labilization of the C—O—C linkage by insertion of an oxygen atom at one of the O-linked carbons to generate a hemiacetal. Because hemiacetals dissociate rapidly in aqueous solution, the oxygenase enzymes effectively predispose the molecule to a spontaneous dissociation of the erstwhile stable ether linkage. Schink et al. (94) speculated that such oxygenase-mediated scission via hemiacetal formation should enable the depolymerization of the larger polyethers.

Oxidation by Cytochromes P-450

Microbial cytochromes P-450 are widely distributed, are known to accept exotic compounds as substrates, and perform a multitude of xenobiotic conversions (92). It is not surprising, therefore, to find their involvement in the scission of ether bonds. During the screening of microorganisms for possession of enzymes that mediate the cleavage of ether bonds of simple aromatic ethers, Karlson et al. (50) isolated a soil organism (subsequently designated a strain of *Rhodococcus rhodochrous*) with the capacity to grow on 2-ethoxyphenol and 4-methoxybenzoate. These two growth substrates induced two separate cytochromes P-450, each performing the O dealkylation

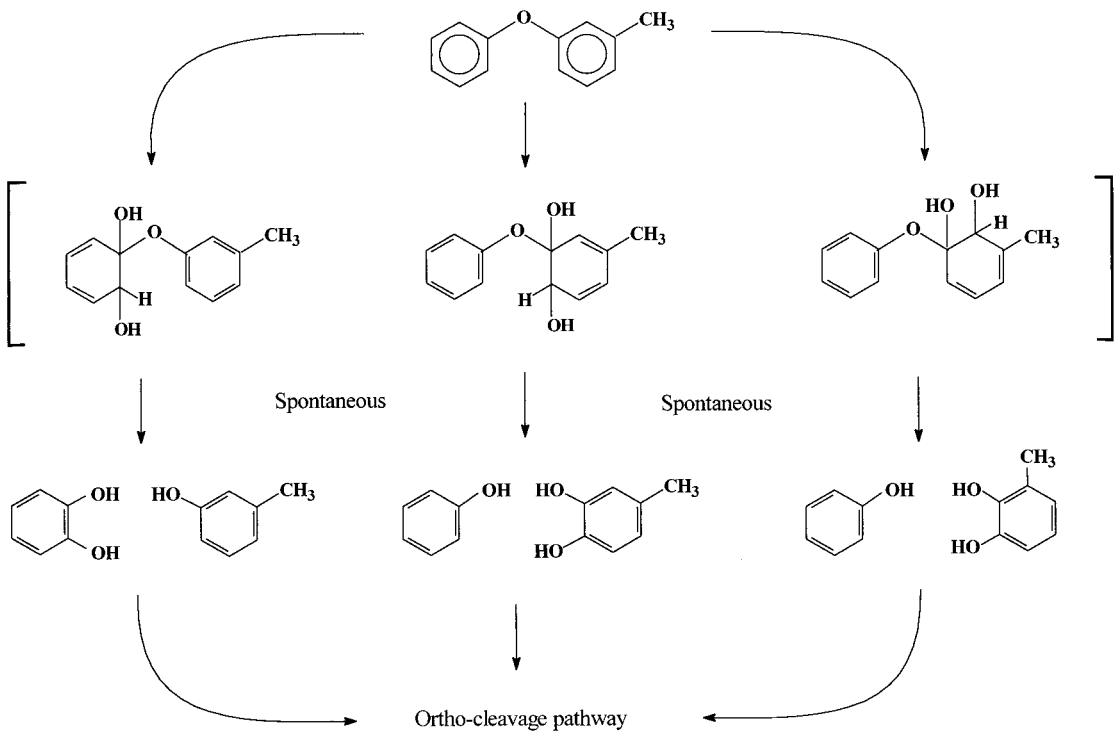

FIG. 3. Ether cleavage steps in the biodegradation of a diaryl ether by dioxygenolytic scission in a *Sphingomonas* sp. Reprinted from reference 97 with permission of the publisher.

of their respective growth substrates (Fig. 4). The soluble cytochrome P-450$_{RR1}$ induced during growth on 2-ethoxyphenol was of a size (44.5 kDa per subunit) and form typical of other bacterial cytochromes P-450 and could O dealkylate both 2-ethoxyphenol and 2-methoxyphenol to catechol (23). Cytochromes P-450 which catalyze the O dealkylation of phenolic ethers have been found in several other genera including *Nocardia* (13) and *Streptomyces* (93, 111). Growth of *Streptomyces griseus* on soyabean flour induced a cytochrome P-450 (93) whose characteristics were typical of previously reported cytochromes P-450 which oxidatively O dealkylated 7-ethoxycoumarin to 7-hydroxycoumarin (118). Both veratrole (1,2-dimethoxybenzene) and guaiacol (1-hydroxy-2-methoxybenzene) also induced the production of a cytochrome P-450 in *Streptomyces setonii* which appears to be solely responsible for the O demethylation of these compounds (Fig. 5) (111).

While the nature of the detected products shows that removal of alkyl groups has occurred, the mechanistic details of these reactions remain obscure. For example, there is no direct evidence from which to deduce the chemical mechanism by which the ether cleavage occurs. Indeed, it is not even known whether the aryl-O or the alkyl-O bond is broken. It is tacitly assumed that in keeping with the widely established oxidative function of cytochromes P-450, the monooxygenase activity of the cytochrome P-450 complexes achieves hydroxylation of the alkyl group. In the methyl-aryl ethers (23, 50, 111), hydroxyla-

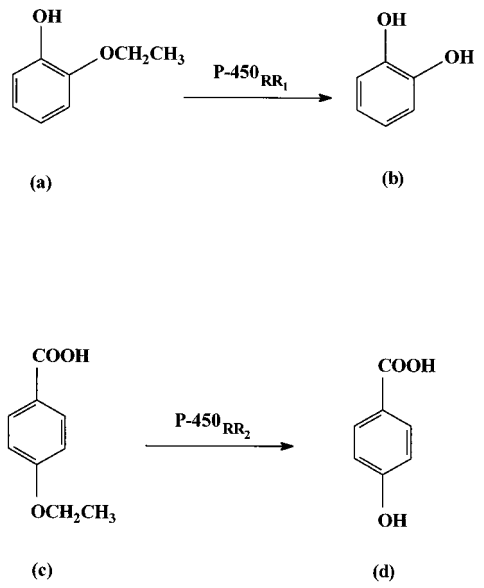

FIG. 4. Conversion of 2-ethoxyphenol (a) to catechol (b) and 4-ethoxybenzoate (c) to 4-hydroxybenzoate (d) by cytochromes P-450 from *R. rhodochrous*. Adapted from reference 50.

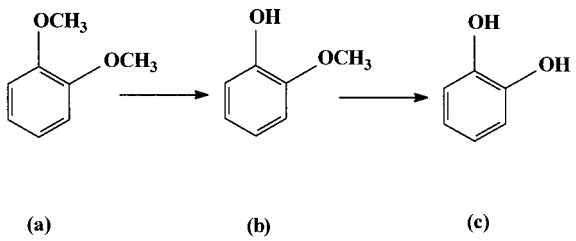

FIG. 5. Example of methyl-aryl ether cleavage mediated by cytochrome P-450. Shown is dealkylation of veratrole (a) to guaiacol (b) and thence to catechol (c) by cytochrome P-450 from *Streptomyces setonii*. Adapted from reference 111.

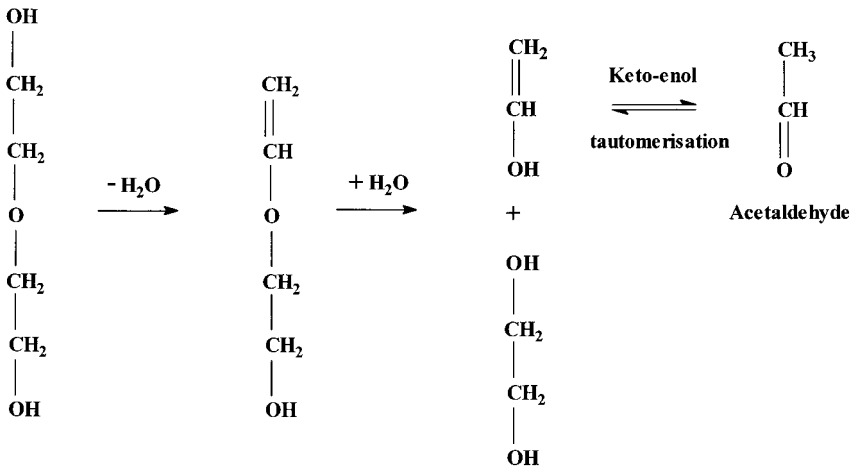

FIG. 6. The vinyl-ether hydrolysis mechanism of diethylene glycol cleavage into ethylene glycol and acetaldehyde by an *Acinetobacter* sp., proposed by Pearce and Heydeman (85). Reprinted with permission of the publisher.

tion of the ether-linked methyl group would produce a hemiacetal, spontaneous cleavage of which would yield formaldehyde and phenol. For 2-ethoxy derivatives (23, 50, 93), the corresponding product would be acetaldehyde. However, it is not possible at present to substantiate this mechanism, because there are no published data on the chemical form in which the alkyl group is liberated from its ether linkage by cytochrome P-450 systems. Moreover, the cytochrome P-450 in *R. rhodochrous* may direct attack not at the ether-linked carbon, as described above, but at the terminal CH_3 group. While this is one and the same carbon atom for methoxy phenols, in ethoxy phenols such an oxidation would produce initially 2-(2′-hydroxyethoxy)phenol (ϕ-O-$CH_2 \cdot CH_2OH$, i.e., a glycol-substituted phenol). Migration of the terminal OH group to the ether-linked carbon (a mechanism known to occur in other ether-cleaving systems [see below]) would produce the labile hemiacetal precursor of ether cleavage. Data are not yet available to distinguish between these mechanisms.

The substrates considered thus far are alkyl-aryl ethers, and, as pointed out above, π-bonding strengthens the aryl-O bond in comparison with the alkyl-O bond, rendering the former less susceptible to scission. However, there is evidence that cytochrome P-450-initiated scission of the aryl-O bond (sometimes referred to as dealkoxylation) can occur in aryl-alkyl ethers in mammalian systems (80). In this system, the aryl group to be severed from the ether oxygen must contain a phenolic OH group to facilitate its oxidation by the cytochrome P-450 system to a benzoquinone product. Provided that the aryl group is phenolic (e.g., 4-methoxyphenol), aryl-O fission occurs in preference to dealkylation. The so-called *ipso*-substitution pathway involves insertion of a hydroxyl at the ether-linked aryl carbon to produce a hemiketal based on a benzoquinone structure. This decomposes to benzoquinone via an aryl-O cleavage with elimination of the intact alkoxy unit. Whether a similar dealkoxylation of alkyl-aryl ethers occurs in prokaryotes remains to be determined.

Hydroxyl Shift Mechanisms

In the oxidative mechanisms discussed above, the ether linkage was transformed to the much more labile hemiacetal by introduction of oxygen to one of the ether-linked carbons. Such transformation to hemiacetal can also be achieved without the incorporation of new oxygen, provided that a free hydroxyl group is present on the carbon vicinal to the ether-linked carbon. Such a situation always occurs at the terminal -O-CH_2-CH_2-OH unit of PEG. This concept was first described by Pearce and Heydeman (85) in a mechanism for PEG degradation by an *Acinetobacter* species. This organism achieved biodegradation of PEG by successive removal of ethylene glycol units from the PEG terminal (exo-cleavage). Although the organism is an obligate aerobe, the process was independent of oxygen, and C_2 units were removed at the oxidation level of acetaldehyde, which in its hydrated form, $CH_3CH(OH)_2$, is equivalent to ethylene glycol. The nonoxidative mechanism proposed by Pearce and Heydeman involved a two-step reaction initiated by a dehydration to produce a vinyl ether intermediate, which was then cleaved in a single hydrolysis step to the enol form of acetaldehyde (Fig. 6). The possible alternative second stage in which the vinyl ether was rehydrated to produce the labile hemiacetal was apparently not considered. In the absence of evidence for the vinyl intermediate and in the light of more recent work (see below), the precise nature of the ether cleavage step (hydrolysis of vinyl ether or aldol cleavage of hemiacetal) in this system remains uncertain. Nevertheless, this work first introduced the important concept of ether cleavage mechanisms which are dependent on the availability of a free OH group at the vicinal carbon, a notion which has since been extensively elaborated for other systems, as will now emerge.

Several strictly anaerobic gram-negative bacteria have been isolated for their ability to ferment PEGs. One of these was a new strain designated *Pelobacter venetianus*, which fermented PEGs intracellularly and without the production of extracellular PEG-depolymerizing enzymes (95). The ether cleavage reaction proceeded in the absence of molecular oxygen, and two possible mechanisms were identified: (i) simple direct hydrolysis of the terminal ether to liberate ethylene glycol and a shortened PEG, and (ii) rearrangement of the terminal R-O-CH_2CH_2-OH unit to the acetaldehyde hemiacetal structure, R-O-CH(OH)-CH_3. The former mechanism is unlikely because of the hydrolytic stability of ether bonds (see the following section). The latter mechanism could be achieved either by dehydration (compare the first stage of the Pearce and Heydeman mechanism [Fig. 6]) and reverse-orientation rehydration or by vitamin B_{12}-dependent shift of the hydroxyl group. In either case, dissociation of the resultant hemiacetal would lead to rapid formation of acetaldehyde and a shortened PEG,

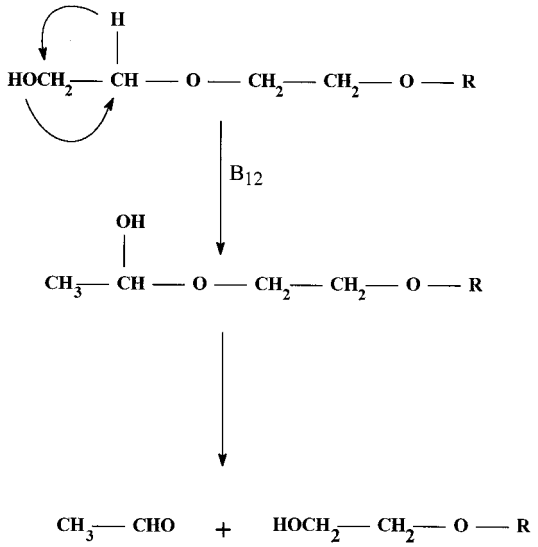

FIG. 7. Vitamin B_{12}-dependent hydroxy-shift mechanism of ether scission analogous to diol dehydratase activity proposed by Frings et al. (28).

which could serve in successive rounds of this exo-cleavage process (Fig. 7). The mechanism involving hemiacetal formation and dismutation was supported by the subsequent demonstration that acetaldehyde was indeed the primary product of PEG degradation in this species (108). Moreover, *P. venetianus* did not degrade tetraethylene glycol dimethyl ether, in which both terminal hydroxy groups are blocked as methyl ethers, but did degrade ethoxyethanol and methoxyethanol (28), which each possess one free OH group. Thus, the presence of one terminal free OH group is necessary and sufficient for PEG degradation in this organism. The further observation that anaerobic enrichments with tetraethylene glycol dimethyl ether did not yield any bacterial growth in more than 3 months suggested that the requirement for a free OH group to facilitate ether scission may hold for other anaerobic bacteria (108).

The enzyme system which liberates acetaldehyde from PEGs has been established in cell extracts of *P. venetianus* (28). The activity was cytoplasmic, stimulated by corrinoids, especially hydroxycobalamin, was highly sensitive to molecular oxygen, and required a low redox potential for activity. The activity was distinct from diol dehydratase (different response to corrinoids and glycerol), which was also present and active in converting ethylene glycol to acetaldehyde. The collective evidence from whole-cell physiological work and the cell-free activity data indicates strongly that PEG degradation proceeds as follows: (i) initial hydroxyl shift from the terminal carbon to the ether-linked carbon, catalyzed by the PEG-degrading enzyme analogous to a vitamin B_{12}-dependent diol dehydratase reaction; and (ii) rapid dissociation of the resulting hemiacetal to acetaldehyde and a shortened PEG. Schink and colleagues (28, 100) have given the name PEG acetaldehyde lyase to the initiating enzyme. An entirely analogous pathway has been reported for the biodegradation of 2-methoxy ethanol in *P. venetianus* (114).

This mechanism has now been implicated in PEG degradation by several other anaerobic bacteria. An analogous PEG acetaldehyde lyase was detected (28) in extracts of *Bacteroides* sp. strain PG1 originally described by Dwyer and Tiedje (21), except that in this case corrinoids were inhibitory. Indeed, Dwyer and Tiedje had already shown that this *Bacteroides* strain converts PEGs to acetaldehyde as a key intermediate. The ability of spent culture medium to convert freshly added PEG 1000 (average molecular weight) into a form assimilable by strains normally unable to utilize intact PEG 1000 was interpreted by Dwyer and Tiedje as indicating that *Bacteroides* cells released a PEG 1000-depolymerizing enzyme. However, in the absence of data to identify and quantify the degradation products, it may be that the growth-supporting components arose from impurities present in the PEG preparation itself, rather than from its depolymerization. In the absence of information about appropriate controls, the growth-supporting components may even be *Bacteriodes*-excreted metabolites or products of cell lysis, quite remote from PEG degradation intermediates.

The failure of *Bacteroides* cell extracts to degrade or dehydrogenate PEG was taken as further evidence in favor of an extracytoplasmic depolymerization of PEG prior to uptake and metabolism of the derived oligomers (21). However, enzyme activities were also not demonstrated in the extracellular medium. A possible explanation for the absence of PEG degradation in the presence of cell extracts may simply be the instability of the enzymes involved. For example, Frings et al. (28) showed that extracts of *P. venetianus* lost 10 to 30% of PEG acetaldehyde lyase activity per h and that the PEG degradation rate was linear for only the initial 1 to 2 min of incubation. Instability has in fact proved so severe as to preclude purification of this most intriguing enzyme. Notwithstanding the lack of strong evidence for extracellular depolymerization, the work of Dwyer and Tiedje is otherwise consistent with an OH shift to form a hemiacetal and its subsequent dissociation as the mechanism for PEG degradation in *Bacteroides* species, and Frings et al. (28) have concluded that despite differences in corrinoid response, this strategy is common to both *Pelobacter* and *Bacteroides* species.

An important residual question raised by Tiedje and Dwyer (21) remains valid (100); namely, "How do the large PEG molecules gain access to the cytoplasmic PEG acetaldehyde lyase enzymes?" Schmid et al. (96) searched without success for a strong interaction between PEGs and porins isolated from the active PEG degrader *P. venetianus*. PEG 20000 had no effect on the specific conductance of membranes reconstituted from a 23-kDa porin protein and a phospholipid bilayer; diethylene glycol and PEG 200 had only weak effects (<10% inhibition of conductance). However, reconstituted membrane systems suffer from the disadvantage that the porins may not orientate and function properly without the correct interactions with the lipopolysaccharide which makes up the lipid in the outer leaflet of the outer membrane in gram-negative bacteria. Thus, the evidence for noninvolvement of porins in PEG uptake is very limited, and the problem remains unresolved.

In another study, *Acetobacterium malicum* grew anaerobically by acetogenic fermentation of 2-methoxyethanol or 2-ethoxyethanol (114) but not of 1,2-dimethoxyethanol. The apparent requirement for a free terminal OH group is reminiscent of the situation described by Schink and coworkers (28, 108) and is consistent with a OH shift-mediated formation of a hemiacetal. This conclusion is strengthened by the detection of acetaldehyde as a reaction product of PEG degradation in the presence of cell extracts of *Acetobacterium* sp. (100). Similarly, extracts of a strictly anaerobic gram-positive rod, also assigned to the genus *Acetobacterium* and growing fermentatively on phenoxyethanol, cleaved the ether linkage to produce acetaldehyde, suggestive once more of a hydroxyl shift to the subterminal carbon and dismutation of the resulting hemiacetal (27).

Finally, coming full circle to the *Acinetobacter* sp. (85) fea-

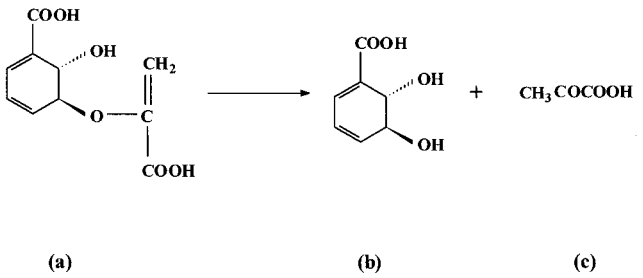

FIG. 8. Conversion of isochorismic acid (a) into 2,3-dihydro-2,3-dihydroxybenzoic acid (b) and pyruvate (c) by isochorismate pyruvate-hydrolyase, (EC 3.3.2.1) from *Aerobacter aerogenes*. Adapted from reference 130.

tured at the beginning of this section, it is worth noting that the PEG-degrading activity in crude extracts was, like the *P. venetianus* PEG acetaldehyde lyase, stimulated by addition of the corrinoids cyanocobalamin and adenosylcobalamin. This finding suggests that the ether-cleaving mechanisms in these organisms bear a closer similarity than would be indicated by comparison of those already proposed (Fig. 6 and 7). A reappraisal of the Pearce and Heydeman data (85) shows them to be entirely consistent with rearrangement to, and subsequent dissociation of, a hemiacetal rather than with formation of the vinyl ether intermediate which was proposed originally.

Hydrolysis

Ethers are, by comparison with esters, highly stable to hydrolysis even in the presence of mild acids or bases. Thus, it is not surprising that enzyme-catalyzed hydrolysis is an equally rare event. Haines and Alexander (39) suggested that the biodegradation of high-molecular-weight PEGs (PEG 20000) by *Pseudomonas aeruginosa* was initiated by an extracellular depolymerizing enzyme which cleaved PEG hydrolytically. This mechanism was based on a bioassay in which extracellular protein was isolated and incubated with PEG 20000 and the extent of depolymerization was assessed by measuring O_2 uptake by washed whole cells subsequently added to the "depolymerized" PEG. Unfortunately, this approach is complicated by several factors; for example, the organism was enriched on PEG and so may have oxidized intact PEG as well as any degradation products. In addition, commercial PEGs are mixtures and the O_2 uptake may reflect oxidation of low-molecular-weight materials already present. Indeed, the authors acknowledge that PEGs from different commercial sources were utilized at different rates even though the mean molecular weights were nominally identical. These results have never been reproduced, and, unfortunately, the strain has been lost, so that the currently weak evidence for hydrolytic depolymerization in this system cannot be substantiated.

A second contender for hydrolytic cleavage of ethers is isochorismate pyruvate-hydrolase (EC 3.3.2.1), distinguished as a participant in the biosynthesis of the growth factor 2,3-dihydroxybenzoic acid (129, 130) in *Aerobacter aerogenes*. In the absence of added cofactors, this enzyme catalyzed the conversion of isochorismic acid to 2,3-dihydro-2,3-dihydroxybenzoic acid by an apparently hydrolytic ether cleavage mechanism (Fig. 8). No data are available from which to deduce a precise mechanism for this reaction. A direct nucleophilic displacement by water at either the aryl-O or alkyl-O bond is possible, liberating the phenol and the enol form of pyruvate. This would be analogous to the hydrolysis of a vinyl ether intermediate (Fig. 6) proposed for PEG degradation (85). Alternatively, the enzyme may catalyze hydration of the double bond, thus introducing an OH group at the ether-linked carbon and generating a hemiacetal structure. This would dismutate into the keto form of pyruvate with elimination of the phenolate ion. In either case, the cleavage is effected by water and is thus a hydrolysis. However, the latter mechanism is very reminiscent of the hemiacetal formation by dehydration-rehydration to achieve a $\beta \rightarrow \alpha$ hydroxyl shift, except that in this case a double bond is already present and the only enzymatic step required is rehydration. Therefore, whether this reaction should be described as hydrolysis or included with the other hydroxyl shift mechanisms is a moot point.

Anaerobic O Dealkylation of Alkyl-Aryl Ethers

Homoacetogenic bacteria capable of anaerobic growth on methyl-aryl ethers (methoxyaromatics) have been isolated from a variety of anoxic habitats including sewage sludge, sediments, and soil. These compounds, which arise from natural sources such as peat, lignin, and coal and also in pulp and paper mill effluents, provide C_1 units from the ether-linked methyl groups to support growth of a diverse group of facultatively and obligately anaerobic bacteria (19).

The first step in utilization of the C_1 group involves ether scission of the parent methyl-aryl ethers to the corresponding hydroxyaromatic compound. During the early exploration of this process in the 1980s, this reaction was referred to both as demethylation and as demethoxylation. Strictly speaking, these terms hold mechanistic implications in that they indicate methyl-O and aryl-O cleavage, respectively. However, those early studies contained insufficient evidence to assign either mechanism with confidence. Ether cleavage in methyl-aryl ethers giving access to the growth-supporting methyl group was first observed in *Acetobacterium woodii* (3). The process was described as demethoxylation, but the term was used loosely because at that time there was no evidence for the implied aryl-O cleavage. Demethoxylation, transmethoxylation, and hydrolysis have all been considered, but no clear mechanism emerged until the definitive study of DeWeerd et al. (19), using compounds labeled with ^{18}O in the ether oxygen, showed conclusively that the methyl-O bond was broken, leaving the ^{18}O atom still attached to the aryl nucleus. Moreover, the results suggested that methanol might not be the initial C_1 intermediate, a conclusion reached independently by Daniel et al. for another gram-positive anaerobe, a *Clostridium* sp. (18), and by Frazer and Young (26) for an obligately anaerobic gram-negative bacterium.

The notion that ether cleavage involved not hydrolytic demethylation but, rather, a transmethylation to an acceptor other than water was strengthened by the study of Doré and Bryant (20), which implicated the involvement of tetrahydrofolate (a known C_1 carrier) in anaerobic methyl-aryl ether metabolism in *Syntrophococcus* spp. The O-demethylating activities in *A. woodii* (5) and *Sporomusa ovata* (110) have now been confirmed as being strictly reliant upon the presence of tetrahydrofolate. In the latter case, the requirement for tetrahydrofolate has been shown to be stoichiometric with the extent of demethylation of the substrate. The demethylation of 3,4-[4-*methoxy*-^{14}C]dimethoxybenzoate was catalyzed in vitro by corrinoid-dependent proteins (110) to produce *N*-5-[^{14}C]methyltetrahydrofolate. In recent years, numerous corrinoid-dependent methyl transferases have been reported (109). Many of the reactions are catalyzed by enzymes from bacteria characterized by distinct C_1 metabolism, notably acetogenic bacteria carrying out methyl transfer in both anabolic and catabolic pathways. Tetrahydrofolate functions as the methyl acceptor, and methyl tetrahydrofolate functions as the methyl

donor. Recent elegant studies by ^{13}C nuclear magnetic resonance spectroscopy have also shown unambiguously that the methyl-aryl ether *O*-demethylase in *Clostridium thermoaceticum* transferred the methyl group directly to tetrahydrofolate. The *O*-demethylase was insensitive to oxygen, did not require reductive activation, and was unaffected by propyl iodide. Thus, in contrast to the *Sporomusa ovata* system, the *O*-demethylase in *C. thermoaceticum* was not itself a corrinoid protein, although subsequent enzymes in the acetogenic pathway were corrinoid dependent.

Clarification of the corrinoid dependence of *O*-demethylation has been achieved in studies with *Holophaga foetida* TMBS4, isolated by Kreft and Schink (62). The complete methyl transfer from phenyl methyl ether to tetrahydrofolate is catalyzed by at least two proteins. The overall system required reductive activation, which was stimulated by ATP, and, on the basis of inhibition by propyl iodide, it was corrinoid dependent. Using spectrophotometric analyses, the same authors resolved the two methyl transfer steps which constitute the complete tetrahydrofolate-dependent O-demethylation reaction (63). In the first step, the methyl group was transferred from its ether linkage to enzyme-bound reduced corrinoid to give a methyl corrinoid and thence in the second step to tetrahydrofolate. Thus, the true ether cleavage reaction was the first step; the second step, which transfers the methyl to tetrahydrofolate, may be regarded as part of the onward metabolism of the C_1 moiety. The first reaction (but not the second) was corrinoid dependent. Cob(I)alamin, but not cob(II)alamin, was able to accept methyl groups and to activate the enzyme by reduction, implying that the reaction mechanism was a nucleophilic attack of an enzyme-bound corrinoid in the reduced Co(I) state on the methyl carbon in the methyl-aryl ether.

Although this work relegates tetrahydrofolate to the role of downstream methyl acceptor, not directly involved in ether cleavage itself, involvement of tetrahydrofolate in overall ether cleavage is essential as a sink for methyl groups which otherwise will remain attached to demethylase protein.

The evidence available to date points strongly toward methyl-O scission of the ether linkage, with subsequent transfer of the methyl group to tetrahydrofolate as carrier prior to its incorporation in the acetogenic pathway of energy metabolism. Despite this consensus, there remain several apparent differences among species so far studied. For example, there are differences in the involvement of corrinoids, which is well established in *Sporomusa ovata* and is achieving a level of understanding in *H. foetida*, but apparently does not feature in O demethylation in *C. thermoaceticum*. Equally, differences exist in the need for ATP in the transmethylation reaction. In *C. thermoaceticum*, there is an established stoichiometric requirement for ATP (52), whereas in *Sporomusa ovata* (110) and *H. foetida* (63), substoichiometric amounts of ATP are sufficient to activate the transmethylation system. The demand for ATP in *A. woodii* has been insufficiently studied to permit any conclusion to be drawn.

Oxidation to Carboxylic Acids

In a series of reports, Kawai and colleagues have elaborated a PEG biodegradation pathway for a symbiotic mixed culture composed of a *Flavobacterium* sp. and a *Pseudomonas* sp., in which PEG appears to be oxidized before it succumbs to ether cleavage. For depolymerization of PEGs with average molecular weights of 300 to 20,000, the activities of three enzymes, namely, PEG dehydrogenase, aldehyde-oxidizing enzyme, and an ether-cleaving enzyme, were considered to operate in tandem to catalyze the reaction sequence shown in Fig. 9. The

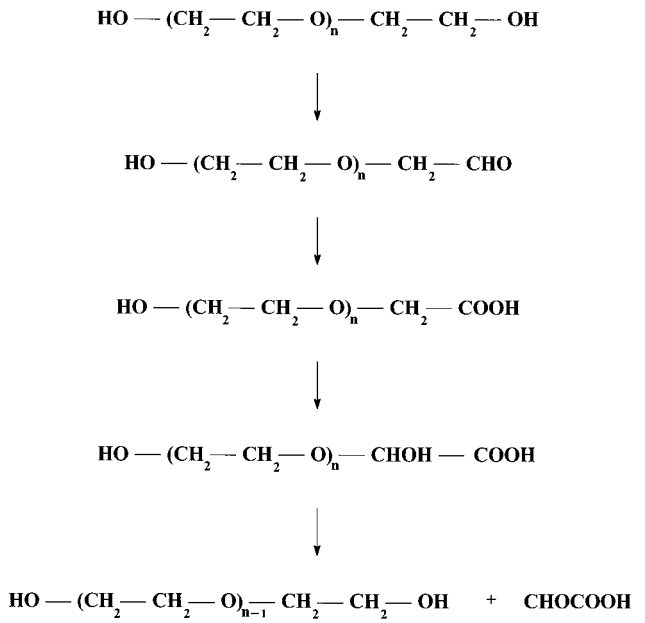

FIG. 9. Oxidative mechanism for ether scission of PEG by a synergistic mixed culture, as proposed by Kawai (53).

PEG dehydrogenase was first detected in the particulate fraction of cell extracts as a PEG-dependent catalyst of DCPIP (dichloroindophenol) reduction, with simultaneous formation of a 2,4-dinitrophenylhydrazine-positive compound, presumed to be an aldehyde (56), although the nature and amounts of the aldehydic material produced were not reported. Whole cells converted tetraethylene glycol to a mixture of triethylene glycol and tetraethylene glycol monocarboxylic acid as "major products," with smaller amounts of tetraethylene glycol dicarboxylic acid, diethylene glycol, and ethylene glycol, although, again, no quantitative data are available. From this information Kawai et al. (56) proposed the pathway depicted in Fig. 9, evidently making the assumption that the accumulation of carboxylic acids indicated their intermediacy in the pathway. This assumption is questionable, because it might equally be argued that oxidized PEGs were accumulated as end products of a nonproductive (in terms of ether cleavage) dead-end pathway. For example, oxidized PEGs might be accepted fortuitously by nonspecific dehydrogenases as a means of regenerating reduced cofactors.

Subsequently, the PEG dehydrogenase was purified and characterized in some detail (57) but, again, without identifying unequivocally the nature of products and stoichiometry of their formation. Consequently, in later studies of the ether cleavage step, Kawai (53) was forced to assume that diglycolic acid was a suitable model compound as the end product of PEG dehydrogenase/aldehyde-oxidizing enzymes and therefore suitable also as a model substrate for the final ether cleavage step. In the presence of cell extracts and DCPIP, diglycolic acid was converted to glyoxylate, but neither the identity of the coproduct nor the stoichiometry of the reaction was established. Tetraethylene glycol dicarboxylic acid was a better substrate than diglycolic acid, and methoxyacetate and ethoxyacetate were weaker substrates. While usefully identifying an ether cleavage reaction, these studies threw little light on the mechanism of the process. Nevertheless, Kawai (53) proposed the new name *O*-decarboxymethylase (ether cleaving) for the enzyme and suggested that, in some unspecified

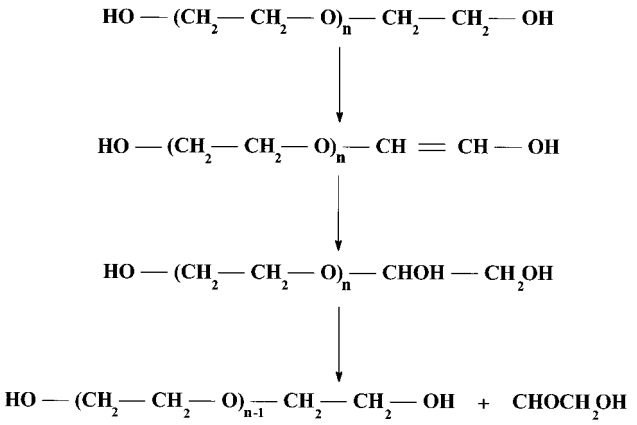

FIG. 10. Dehydrogenation mechanism for ether cleavage in PEGs by *Pseudomonas* sp. strain P 400, as proposed by Thélu et al. (115).

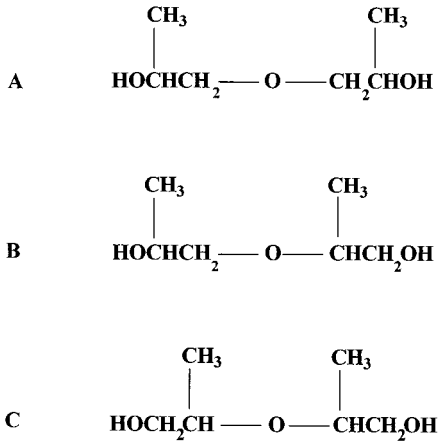

FIG. 11. Structural isomers of dipropylene glycol (see the text for details).

way, it achieved hydroxylation of the ether-linked carbon to the hemiacetal as shown in Fig. 9. While evidence for this mechanism is weak, it is nevertheless consistent with the formation of glyoxylate as one product and with the frequent involvement of the hemiacetal strategy in other ether-cleaving bacteria (see above).

Crude extracts of a *Pseudomonas* sp. isolated from soil by Thélu et al. (115) were shown to reduce DCPIP in the presence of several PEGs, PEG-based nonionic surfactants, or diethylene glycol. PEG oligomers in which one or both terminal hydroxyls were methylated and 2-ethoxyacetate were also electron donors to DCPIP, indicating that oxidation of OH was not the only source of reducing equivalents for DCPIP. Despite the absence of any data on the identities of intermediates or products and largely ignoring the probability that more than one enzyme was involved in the oxidation of these numerous and diverse substrates, a single mechanism (Fig. 10) which involved the transient formation of a double bond was proposed. The evidence for this route is very weak, and its general validity for all the compounds tested is questionable. For example, in the case of PEGs with free OH groups, the transient enol intermediate would be much more likely to arise from oxidation of the alcoholic OH to aldehyde (as occurs in step 1 of the pathway postulated by Kawai and colleagues [Fig. 9]) followed by keto-enol tautomerisation; for 2-ethoxyacetate, the acetate C_2 unit cannot undergo dehydrogenation, implying an unusual dehydrogenation of the CH_3CH_2 unit which could be confirmed by analysis of the product identities.

Influenced by the work of Kawai's group, Obradors and Aguilar (77) searched for and found PEG dehydrogenase in extracts of a PEG-degrading *Pseudomonas stutzeri* strain isolated from river water. Crude cell extracts reduced DCPIP in the presence of PEGs with molecular weights up to about 20,000, diethylene glycol, or diglycolic acid. Because diglycolic acid differs from the other substrates in containing no hydroxyl groups, it is surprising that all of these activities were attributed to a single "PEG dehydrogenase" enzyme in the crude extract (77). The product of the PEG dehydrogenase-catalyzed reaction (acting on an unspecified substrate) was glyoxylate, and on the basis of this weak evidence, the mechanism of Kawai (Fig. 9) was invoked as being the most likely pathway.

Convincing mechanistic data for the ether cleavage biodegradation of PPG are even more sparse. Dipropylene glycol used as a model compound in studies on PPG degradation by a *Coryneform* species (58) contained the three positional isomers shown in Fig. 11, among which there are also numerous diastereoisomers. Despite this complexity, analysis of products of whole-cell biodegradation revealed 1,2-propandiol and two other main metabolites corresponding to the oxidation of one or both of the OH groups to carbonyls. The 1,2-propandiol could arise through a variety of mechanisms for ether cleavage. The oxidized metabolites were taken as evidence for an oxidative mechanism for ether cleavage. However, for reasons described above, accumulation does not necessarily imply intermediacy in the assimilation pathway; it could equally mean that they are dead-end products of a parallel pathway.

Several other authors have also demonstrated the occurrence of PEG oxidation (46, 54, 84, 115, 128), but its relevance to the ether cleavage step in PEG biodegradation is still not convincingly established and a number of points remain unresolved. First, the reaction sequence proposed would be much more convincing if there existed clear stoichiometric relationships between substrate consumed and product formed, especially for the first (PEG dehydrogenase) and third (ether-cleaving) steps. Second, the absence of quantitative data on the overall flux of PEG through this route makes it impossible to judge whether this is the sole pathway in the respective cultures or whether it is only one contributor among several parallel pathways. For example, it is conceivable that the oxidized compounds are not in the PEG assimilation pathway but arise from a competing dead-end pathway. Third, the use of redox dye-based assays with crude extracts can be misleading, especially when the assay has not been validated by correlating the amounts of substrate converted or product formed with the extent of dye reduction. Crude cell extracts contain multiple activities which can utilize DCPIP as the electron acceptor. For example, DCPIP assays with PEG in crude extracts (56) were used to measure PEG dehydrogenase activity, but, clearly, the ether-cleaving and possibly the aldehyde-oxidizing enzymes would also have contributed to DCPIP reduction under these conditions.

Reduction

Although relatively few reports propose the involvement of reductive mechanisms in the cleavage of ether bonds, there is evidence for their existence. Contrary to an earlier report (16) demonstrating an oxidative cleavage of the arylglycerol-β-aryl ether linkage by a *Pseudomonas* sp., cell extracts of *Pseudomonas paucimobilis* SYK-6 contained such a β-etherase activity, which was stimulated by NADH (71). This was mediated by an

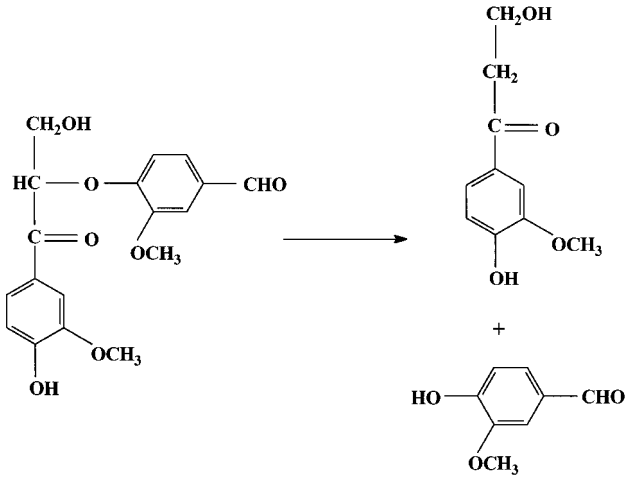

FIG. 12. Reductive cleavage of the arylglycerol–β-aryl ether linkage by *Pseudomonas paucimobilis*, as proposed by Masai et al. (71). Reprinted with permission of the publisher.

inducible, membrane-associated enzyme (LigE) which exhibited a narrow substrate specificity requiring the presence of a carbonyl group adjacent to the ether-linked carbon (Fig. 12); substrates which contained a hydroxyl group at this position were cleaved only following oxidation to the carbonyl derivative. Further investigation (70) into the β-etherase activity of *P. paucimobilis* SYK-6 distinguished an additional enzyme (LigF), which facilitated the reductive scission of the ether bond. Both the LigE and LigF enzymes displayed sequence homology with glutathione-*S*-transferase, and their rates of ether scission increased 2- and 15-fold, respectively, in the presence of 2 mM reduced glutathione. Masai et al. (70) conjectured that there were two similar but independent β-etherase enzymes whose reductive cleavage of the arylglycerol–β-aryl bonds required reduced glutathione as a source of reducing equivalents. A separate NAD(P)H-dependent glutathione reductase activity was also detected in *P. paucimobilis* SYK-6. A possible role of the essential carbonyl group is to generate an electrophilic center at the ether-linked carbon to prepare it for attack by an appropriate reducing agent. However, the precise details of the cleavage step and the involvement of glutathione remain unknown.

Carbon-Oxygen Bond Cleavage by Lyases

In response to environmental concerns about the detergent builder CMOS, a study of its biodegradation by a *Zoogloea* sp. revealed a novel C—O lyase activity in crude cell extracts (87). Oxidation and hydroxylation mechanisms were eliminated on the grounds of lack of requirement for O_2 and inability of redox cofactors (NAD^+, FAD^+, methylene blue) to affect the kinetics. When cell extracts and CMOS ^{14}C labelled in both the carboxymethyl and succinate moieties were used, radiolabelled glycolate and fumarate were the major metabolites during the early stages of incubation, with later production of malate as the accumulation of fumarate (but not glycolate) slowed. The molar sum of fumarate plus glycolate always equalled the amount of CMOS degraded, and the recovery of radiolabel was always around 100%, indicating the absence of alternate major pathways. The progressive accumulation of glycolate, together with the precursor-product relationship between fumarate and malate, provided strong evidence that glycolate and fumarate were initial coproducts of ether cleavage. A

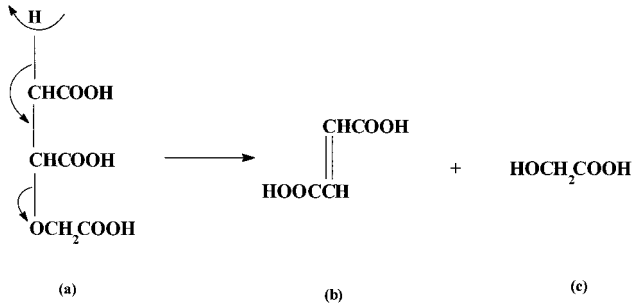

FIG. 13. Carbon-oxygen lyase-mediated cleavage of CMOS (a) into fumaric acid (b) and glycolic acid (c).

carbon-oxygen lyase enzyme (EC 4.2.99.12) was proposed to catalyze the β-elimination reaction, as shown in Fig. 13.

CONCLUDING REMARKS

State of Knowledge and Prospects

Pathways. Almost every conceivable mechanism has been invoked, albeit with different degrees of justification, to account for the ability of bacteria to break ether bonds. The list includes oxygenation, dehydrogenation, hydroxyl group transfers, hydrolysis, reduction, nucleophilic substitution, and dismutation. For some of these, there is compelling evidence to support proposed pathways (e.g., monooxygenation in aerobic degradation of methyl-aryl ethers, hydroxyl shift mechanisms in the anaerobic degradation of PEGs, methyl transfer to tetrahydrofolate in anaerobic degradation of methyl-aryl ethers, and carbon-oxygen lyase in degradation of CMOS), whereas for other pathways, the evidence is more fragmentary. For example, while there is abundant evidence for the occurrence of PEG oxidation, it is by no means firmly established that this is a prerequisite for a major route leading to ether cleavage. Similarly, cytochrome P-450-mediated demethylation of methyl-aryl ethers is assumed to proceed by alkyl-O scission, but data identifying the C_1 products are lacking. The prospect of glutathione involvement in a reductive cleavage of aryl ethers seems quite plausible, especially in view of the ubiquity of glutathione *S*-transferase reactions, but unequivocal evidence for such involvement and certainly for defining a detailed mechanism are needed.

For metabolic pathways of ether degradation to be clearly defined, it is necessary to establish not only the identities of intermediates and products but also the stoichiometric relationships among them and with the substrates utilized. This allows identification of chemical processes linking the substrate and metabolites and thereby guides the design of experiments to probe the mechanisms involved. Moreover, quantitative analysis of intermediates and products is needed to provide reassurance (or otherwise) that the pathway(s) proposed accounts for all the substrate utilized and that alternate pathways are not overlooked. Application of isotopic tracer techniques, including substrates labelled with ^{18}O in the ether oxygen or labelled with ^{14}C in the ether-linked moieties, coupled with appropriate thin-layer chromatography, gas-liquid chromatography, or high-pressure liquid chromatography analyses have already been applied successfully in metabolic studies, and their wider application should help to resolve some of the outstanding questions. In addition, the use of ^{13}C nuclear magnetic resonance spectroscopy to identify and monitor metabolite production in real time in vivo can be especially help-

ful when it proves difficult to establish degradative processes in cell extracts, but application of the technique may be restricted by the unavailability of ^{13}C-labelled substrate and/or lack of sensitivity.

In some of the pathways proposed hitherto, ultimate degradation of ethers appears to require several steps (e.g., oxidation of PEGs to aldehydes and then to carboxylic acids prior to ether cleavage [see the section on oxidation, above]). To dissect such pathways, selection and characterization of mutants deficient in enzymes for component reactions will help to confirm the existence of individual steps in given pathways and the relative importance of potential multiple pathways. Whatever methods are to be used, much work remains to be done in establishing unequivocally the detailed metabolic pathways for ether cleavage in the majority of systems so far identified.

Enzymes. Very few enzymes involved in ether cleavage have been purified and characterized, and despite the apparently rich mix of mechanisms involved in ether catabolism, only three ether cleavage enzymes are accredited with a formal listing (99, 123), namely, isochorismate pyruvate lyase (EC 3.3.2.1), carboxymethyloxysuccinate lyase (EC 4.2.99.12), and 4-methoxybenzoate monooxygenase (EC 1.14.99.15). There appears now to be sufficient evidence to warrant the inclusion of a fourth enzyme in this list, namely, the PEG acetaldehyde lyase described by Schink and colleagues (28, 100), under the carbon-oxygen lyase group (EC 4.2.99).

As the preceding paragraphs have described, many ether cleavage systems convert substrate to a hemiacetal structure, which then undergoes dissociation to break the erstwhile ether link, probably via a nonenzymatic step (see below). In these cases, it is appropriate to focus on the enzyme(s) which catalyze hemiacetal production, but, unfortunately, none of these has yet been purified. While some progress can be (and has been) made with crude extracts, full characterization of enzyme properties requires the use of purified proteins to unravel complexities of mechanism and regulation, especially in multiple-step pathways in which other enzymatic reactions may interfere with the analysis and confuse the picture. Part of the difficulty in this area is the apparent instability of ether-cleaving enzyme systems. In addition, multiple-component enzyme systems may well be involved. Thus, although there is an outstanding need for purification and characterization of ether-cleaving enzyme systems, this is not a task to be undertaken lightly.

Emerging Themes

The need for eight mechanistic headings to structure this review suggests that ether cleavage is mediated by a heterogeneous collection of enzymes and pathways. While the enzymatic machinery may well be diverse, some unifying themes in the strategy of bacterial assimilation of ethers are now beginning to emerge. Of these, the most pronounced is the formation of a hemiacetal structure as the penultimate step in the ether cleavage process. Although the strength and quality of evidence vary, this holds for oxygenases acting on dialkyl ethers, alkyl-aryl ethers, and diaryl ethers and for hydroxyl shift in anaerobic degradation of PEGs. Hemiacetal formation is also strongly implied in, although not yet proven for, mechanisms in which oxidation is achieved by bacterial cytochrome P-450. In addition, mechanisms postulating hydrolysis of vinyl ethers in the aerobic degradation of PEGs (85) and in the biosynthesis of 2,3-dihydroxybenzoate from isochorismate (129, 130) may in fact occur via hydration of the double bond to the hemiacetal, in a manner similar to that proposed by Thélu et al. (115) for oxidative biodegradation of PEGs (Fig. 10). Once formed, the hemiacetal is cleaved via an intramolecular rearrangement which is essentially a β-elimination, not involving water; some authors have referred to it erroneously as a hydrolysis. Interestingly the ether cleavage in CMOS also occurs by a β-elimination (Fig. 13) although not of a hemiacetal. In this case, the elimination generates a C═C double bond (as fumarate) rather than the aldehydic C═O bond, but the similarity of these processes is obvious (Fig. 14).

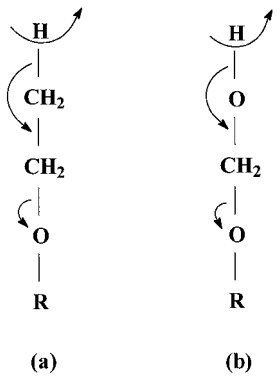

FIG. 14. β-Elimination reactions in the cleavage of ethers. Eliminations across a C—C bond as in CMOS lyase (a) and C—O bond in the decomposition of hemiacetals (b) are shown.

Among the reactions not conforming to the hemiacetal pathway, nucleophilic displacement appears to be an alternative strategy. Thus, the dependence of the reductive cleavage of arylglycerol-β-aryl ether on the presence of an electrophilic carbonyl group adjacent to the site of cleavage (Fig. 12) and the methyl transfer to enzyme-bound reduced corrinoid are both consistent with nucleophilic displacement reactions.

A third feature to emerge is the dependence of some ether cleavage systems on corrinoids, for example, in the HO shift mechanisms for formation of hemiacetals in anaerobic degradation of PEGs and in mediation of methyl group transfer to tetrahydrofolate in the anaerobic demethylation of methyl-aryl ethers. Comparison of the role of the corrinoids in the respective systems must await further study and is likely to be a fruitful avenue of research.

Natural Substrates for Ether Cleavage Systems

Studies on the biodegradation of alkyl-aryl ethers have usually been undertaken in the context of biodegradation of lignin (see, e.g., references 5, 13, 17, 19, 28, 52, 70, 78, 105, and 131), the plant material which is the most abundant polyaromatic and the second most abundant polymer (next to cellulose) in the biosphere. In broad terms, the depolymerization of lignin is an oxidative process achieved predominantly by aerobic fungi (78), which leads to the production of numerous oligomeric and monomeric aromatics including alkyl-aryl ethers, which serve as potential substrates for bacterial ether cleavage systems. As described in preceding sections, dealkylation of alkyl-aryl ethers may occur in aerobic and anaerobic bacteria. Evolution of these catalytic activities for alkyl-aryl scission can thus be seen as a response to exposure to natural compounds throughout evolutionary time rather than as a relatively short-term response to industrial pollutants.

In contrast, much of the work on the biodegradation of aliphatic ethers reflects concern about the fate and possible detrimental effects of industrial compounds such as PEGs and nonionic surfactants following release into the environment. Scant regard has been paid to the role which naturally occur-

ring aliphatic ethers may have played in stimulating the evolution of biodegradative pathways in times long before the current industrial age. Evidence for the occurrence of such compounds is now emerging in the form of ether lipids in bacteria.

There are several types of ether lipid in bacteria. Plasmalogens are found in relatively large proportions in the lipids of anaerobic bacteria, including those of the rumen; these lipids have an ether-linked alkyl chain at the *sn*-1 position and an ester-linked acyl chain at the *sn*-2 position. The plasmalogen forms of phosphatidylethanolamine and phosphatidylglycerol and its derivatives are the most important. Other bacteria contain large amounts of nonplasmalogenic dialkyl and monoalkyl/monoacyl phospholipids, as well as the more usual ester-linked diacyl phospholipids. Recently, the extremely thermophilic, anaerobic, sulfate-reducing bacterium *Thermodesulfotobacterium commune* has also been shown to contain largely diether-linked phospholipids and glycolipids with mainly *br*17:0 alkyl chains. This organism is found in the same extreme environment as some members of the *Archaea*, which also contain ether lipids.

The glycerol ether lipids of *Archaea* members contain only phytanyl (saturated C_{20} or C_{25} isoprenoid-derived) alkyl chains which are linked to glycerol carbons in the *sn*-2,3 configuration, compared with the usual *sn*-1,2 configuration of comparable lipids in bacteria and all other organisms (30). The various types of archaeal lipids include diphytanylglycerol diether analogs of phosphatidylglycerol, phosphatidylglycerolphosphate, and phosphatidylglycerolsulfate, which are the major lipids in extreme halophiles, as well as a number of glycosylated diphytanylglycerol diethers and dibiphytanyldiglycerol tetraethers. The tetraethers are the predominant lipids in thermoacidophiles and thermoalkaliphiles, whereas methanogens contain a mixture of di- and tetraethers. The thermoacidophiles also contain, for example, a calditolglycerol tetraether lipid in which one glycerol is replaced by a C_9 polyol named calditol. In some members of the *Archaea*, the phytanyl chains may be unsaturated or may contain up to four five-membered rings per chain (30, 75).

If the study of the occurrence and biosynthesis of ether lipids in bacteria is in its infancy, our knowledge of the mechanisms of the turnover or breakdown is nonexistent by comparison. The ether link is known to be resistant to the action of phospholipases, making liposomes of ether lipids attractive for applications in biotechnology because of their inherent stability (15) and potential cytostatic properties (82). Beyond this rather negative observation, nothing is apparently known about the turnover and biodegradation of ether lipids. Following recent progress on the mechanisms of synthesis of naturally occurring bacterial ether lipids, some attention should now switch to how they are degraded. By analogy with other biological systems, this is likely to provide valuable clues into the biodegradative mechanisms which bacteria use to break down xenobiotic ether compounds.

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